MEASUREMENT OF COPPER-INDUCED STRESS IN *PIMEPHALES PROMELAS* USING GENE EXPRESSION PROFILES

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Abstract

We are characterizing the gene expression profiles of larval Pimephales promelas in response to varying doses of copper stress. Up to 48-hr old fathead minnow larvae were exposed to copper concentrations of 50 µg/L, 125 µg/L and 200 µg/L over a duration of 48 hours. Survivors from the treated and control groups were then collected in duplicate, RNA was isolated and analyzed by the differential display technique using fluorescent anchored primers and random arbitrary primers. Forty-one different primer combinations have generated 2197 cDNAs. Two hundred and forty-five of these cDNAs have been collected as up-regulation candidate genes, of which 110 showed concentration-dependence. Three hundred and eighty-two cDNAs were selected as down-regulation candidate genes, 198 of which were repressed at one or both of the higher copper dosages used. One percent of the bands were anomalous, and these could either be artifacts of the PCR or due to genetic polymorphism. At present, all these candidate genes are in the process of being sequenced and some have been identified by homology. Further experiments are in progress utilizing zinc and heat and stressors in order to determine whether the differentially expressed transcripts are copper-specific or are affected by general stress.

Introduction

Differential gene expression has been observed in developmental stages (Geschwind et al., 2001), diseased physiological states (Kim et al., 2001) and in response to different stressors (Denslow et al., 2001; Merchan et al., 2001; Rhodes at el., 1997). We are using the differential display technique in order to obtain candidate genes that respond to copper and other stressors, and will confirm their expression using microarrays. Microarrays can provide valuable insight for assessing gene expression under different environmental conditions, and can be used a base for studying regulatory mechanisms of these genes (Gibson, 2002).

We are interested in separating genes that respond to general stress from genes that are stressor-specific. For this purpose, we are using three different stressors: copper, zinc and elevated temperature. Genes commonly affected by all three stressors will be inferred to be those responding to general stress. We are also testing if gene expression is related to the amount of stress, and are using different treatment levels for each experiment.

We are using *Pimephales promelas* larvae for our first set of experiments. Larvae are the most sensitive stage of fish development and are most vulnerable to environmental perturbation at this life stage. Larvae less than 72 hours old are also able to live on the contents of their yolk sac, and therefore the genome is mainly devoted to survival of the organism until the next developmental stage. Using larvae also provide for whole organism assay without the complications of assessing gene expression in different tissues.

Pimephales promelas is a commonly-used test organism in toxicity tests, and has a wide geographic distribution within the United States of America. It is easily cultured in laboratories and can be induced to spawn indoors throughout the year (Weber, 1993).

Materials and Methods

Copper Stress

- Fathead minnow larvae were stressed for 48 hours using copper concentrations of 50 µg/L, 125 µg/L and 200 µg/L. 25 larvae were placed in a 1-liter beaker containing the copper solution, and solutions were changed after 24 hours.
- Between 100-140 survivors were collected from the control and each of the treatment groups. Larvae were then placed in fresh water, splitting up each treatment group into two populations for subsequent RNA analysis.

Zinc Stress

- Fathead minnow larvae were stressed for 48 hours using zinc concentrations of 200 µg/L, 400 µg/L, 600 µg/L and 900 µg/L.
- Between 100-177 survivors were collected from the control and treatment groups at the end of 48 hours. Survivors were then placed in fresh water.

Thermal Stress

- Fathead minnow larvae will be stressed for 24 hours and 48 hours at a temperature of 30ºC (each treatment will have a control group).
- Survivors will be collected and RNA isolated shortly after collection.

RNA Analysis

- RNA is isolated from each sample using TRI REAGENT (MRC, Inc., Cincinnati, OH) following the manufacturer's protocol with the following modifications. The RNA is precipitated two times with ethanol and then treated with DNAseI (Ambion, Austin, TX) to remove any possible contaminating DNA. The DNAse is removed by adsorption to a proprietary material provided with the DNA-free kit (Ambion, Austin, TX)
- One to two micrograms of RNA are usually recovered per fish. To date, all isolated RNAs had UV 260/280 profiles ~2.0 and gave agarose electrophoresis gel distributions that were characteristic of non-degraded RNA.
- The RNA samples are frozen in aliquots. When needed, samples are defrosted, adjusted to 0.1 µg/µl and copied into cDNAs according to the Genomyx LR (Beckman Coulter, Inc., Fullerton, CA) recommended protocols.
- We are using the FluoroDD HIEROGLYPH Differential Display mRNA Profile Kit (Beckman Coulter Inc., Fullerton, CA) to analyze cDNAs obtained from our experiments. Individual fish larvae from each experimental group were pooled together (minimum *N*=50) for RNA isolation and analysis. This was done in order to obtain sufficient RNA for analysis, as well as to eliminate the confounding effects of individual expression differences.
- The reverse transcription reaction uses a two base anchored poly dT primer that is attached to a T7 promoter (ANCHOR).
- Second strand synthesis is accomplished using AmpliTaq polymerase, a fluorescent T7 anchored primer and a ten-bp arbitrary primer that is attached to an M13 promoter.
- The resulting cDNAs < 500 bp are resolved by electrophoresis for 2.5 hours at 2700 V at 50°C on 33 × 61 cm sequencing gel plates containing 5.6% polyacrylamide.
- cDNAs > 500 bp are resolved for 5 hours at 2700 V at 55°C.
- The fluorescent bands are detected with a GenomyxLR Fluorescent Imaging Scanner (Beckman Coulter Inc., Fullerton, CA) and are stored as tiff images. Examination of the tiff images at high magnification is used to determine if a band's intensity is increased or decreased when compared across the treated and control samples. Bands judged to be differentially expressed (duplicated change within the treatment group) are cut out as candidate genes and stored in buffer.
- cDNAs are cut and purified using the QIAGEN Min Elute Gel Extraction Kit (QIAGEN, Inc.)
- cDNAs are then amplified and sequenced using ABI PRISM BigDye Terminators v3.0 Cycle Sequencing Kit (Applied Biosystems, Inc.) on an ABI PRISM 3100 Sequencer

Results

Copper stress

- Percentage survival from the control group, 50 µg/L, 125 µg/L and 200 µg/L copper treatment groups were 100, 91.2, 68.7 and 56 respectively.
- A total of 2197 cDNA bands were generated using 6 different anchor primers in combination with 3-4 arbitrary primers. Of these, 245 bands were collected as candidates for upregulation with 110 bands showing dosage-dependence.
- Three hundred and eighty-two bands were collected as candidates for downregulated genes in response to copper stress. One hundred and ninety-eight of these showed concentration-dependence.

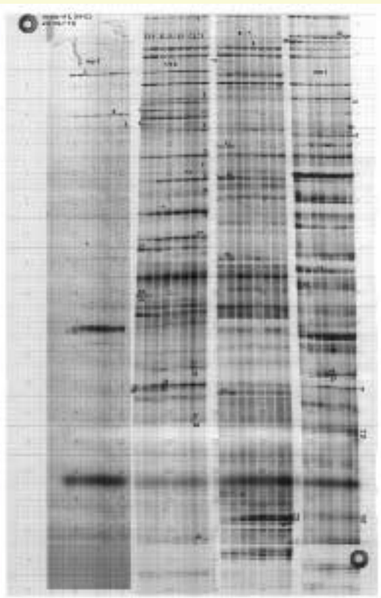

Figure 1 : mRNA isolated from the controls (C1 and C2), 50 µg/L Cu (11 and 12), 125 µg/L Cu (21 and 22) and 200 µg/L Cu (31 and 32) treated populations which were converted to cDNA using Anchor 6 with Arbitrary primers 1,3, 5 and 6.

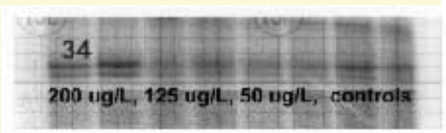

Figure 2: An example of bands whose expression was increased in response to copper. In this case, the elevated intensity of expression is seen only in the 200 µg/L Cu treatment, and not in the samples treated with lower dosages of the metal. The cDNAs were produced using Anchor 6 and Arbitrary primer 3.

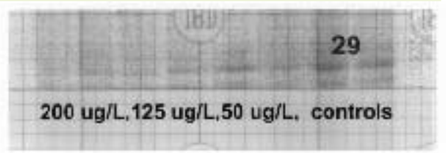

Figure 3: An example of bands whose expression was lowered in response to copper. The cDNAs were produced using Anchor 6 and Arbitrary primer 5.

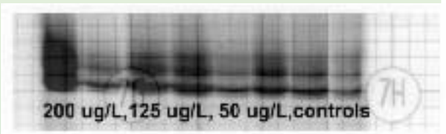

Figure 4: An example of inconsistent expression among the different treatment groups. The cDNAs were produced using Anchor 6 and Arbitrary primer 1.

- Sequencing has been successfully carried out for eleven cDNAs and five of these showed sequence similarity to known sequences in GenBank. The five similar genes are:
  1. "mRNA for elongation factor-1" (*Carassius auratus*, e-139, upregulation candidate)
  2. "CREB binding protein" (*Homo sapiens*, 5e-07, expressed in all groups)
  3. "troponin T skeletal mRNA" (*Danio rerio*, 1e-22, upregulation candidate)
  4. "mRNA from complement C3-H1" (*Cyprinus carpio*, 2e-33, upregulation candidate)
  5. "isolate MOLR19 cytochrome mitochondrial gene" (*Luxilus chrysocephalus*, 1e-55, inconsistent expression)

Zinc stress

Percentage survival from the control group, 200 µg/L, 400 µg/L, 600 µg/L and 900 µg/L zinc treatment groups were 100, 93, 69, 89 and 35 respectively.

- A total of 1274 cDNA bands were generated using 2 different anchor primers in combination with 9 arbitrary primers. Of these, 119 bands were collected as candidates for upregulation with 40 bands showing dosage-dependence.
- Ninety-one bands were collected as candidates for downregulated genes in response to zinc stress. Seventy-two of these bands showed concentration-dependence.

Discussion

Transcription patterns for genetically heterogeneous populations of *Pimephales promelas* in response to copper showed good reproducibility between the two duplicate samples within each treatment. Less than 1% of the bands showed inconsistent expression (19 out of 2197), whereas the 627 differentially expressed bands showed good stability. It is not known whether inconsistent expression of cDNAs is due to the RT-PCR technique or inherent variation in genetic expression among individuals.

Three hundred and eight of the 627 differentially expressed bands showed concentration-dependence, indicating that transcription patterns may be different depending on the level of stress that the organism is subjected to.

The observed differential gene expression has to be confirmed using either gene chips or Northern blots, because this technique has a high false positive rate.

Additional studies using adult fish have to be carried out in order to determine the significance of stress-related genes in the overall ability of a population to overcome stress at different life stages.

Information from all the experiments will provide potential stress-related genes that may have significance in the adaptation of a population to environmental perturbation.

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